Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochimica et Biophysica Acta 1638 (2003) 208–216

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.bba-direct.com

Inhibition of human m-epoxide hydrolase gene expression in a case of hypercholanemia

Qin-shi Zhu[a], Wenxue Xing[a], Bin Qian[a], Patricia von Dippe[a], Benjamin L. Shneider[b], Victor L. Fox[c], Daniel Levy[a,*]

[a] *Department of Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, 2011 Zonal Avenue, Los Angeles, CA 90033, USA*

[b] *Department of Pediatrics, Division of Pediatric Gastroenterology, Nutrition and Liver Disease, Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY 10029, USA*

[c] *Department of Pediatrics, Division of Gastroenterology and Nutrition, Harvard Medical School, The Children's Hospital, Boston, MA 02115, USA*

Received 5 February 2003; received in revised form 1 May 2003; accepted 16 May 2003

Abstract

Microsomal epoxide hydrolase (mEH) is a bifunctional protein that plays a central role in carcinogen metabolism and is also able to mediate the sodium-dependent uptake of bile acids into hepatocytes. Studies have identified a subject (S-1) with extremely elevated serum bile salt levels in the absence of observable hepatocellular injury, suggesting a defect in bile acid uptake. In this individual, mEH protein and mEH mRNA levels were reduced by approximately 95% and 85%, respectively, whereas the expression and amino acid sequence of another bile acid transport protein (NTCP) was unaffected. Sequence analysis of the mEH gene (EPHX1) revealed a point mutation at an upstream HNF-3 site (allele I) and in intron 1 (allele II), which resulted in a significant decrease in EPHX1 promoter activity in transient transfection assays. Gel shift assays using a radiolabeled oligonucleotide from each region resulted in specific transcription factor binding patterns, which were altered in the presence of the mutation. These studies demonstrate that the expression of mEH is greatly reduced in a patient with hypercholanemia, suggesting that mEH participates in sodium-dependent bile acid uptake in human liver where its absence may contribute to the etiology of this disease.

Keywords: Bile acid; Transport; m-epoxide hydrolase; Gene expression

1. Introduction

The bifunctional protein, microsomal epoxide hydrolase (mEH) (EC 3.3.2.3), plays a central role in the metabolism of numerous xenobiotics such as polycyclic aromatic hydrocarbon (PAH) carcinogens in the endoplasmic reticulum [1] where it is expressed with a type I and type II topological orientation [2]. We have previously demonstrated that the type II form is targeted to the hepatocyte plasma membrane [2] where mEH is capable of mediating sodium-dependent uptake of bile acids [3–9] in parallel with the sodium–taurocholate cotransporting protein (NTCP) [10]. The contribution of multiple transporters to the sodium-dependent uptake of bile acids is supported by the observations that in bile duct ligation induced extrahepatic cholestasis in rats where NTCP is undetectable, significant sodium-dependent taurocholic acid transport (30%) is still observed [11] in the presence of normal mEH levels (unpublished observation). The possibility that this residual uptake is mediated by mEH is supported by previous studies using an anti-mEH antibody [9]. Significant transport of taurocholate also occurs in the *fch*/*fch* mouse. In this model of erythropoietic protoporphyria, NTCP as well as a Na^+-independent bile acid transporter, OATP-1, were undetectable, yet 90% of intravenously administered [^{3}H]taurocholate was still transported into the hepatic and biliary compartments [12], indicating an alternative transport system.

To further explore the role of mEH in human bile acid transport, studies have focused on a patient (S-1) who exhibited serum bile salt levels that were elevated approx-

* Corresponding author. Tel.: +1-323-442-1525; fax: +1-323-442-1224.

E-mail address: dlevy@hsc.usc.edu (D. Levy).

0925-4439/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0925-4439(03)00085-1

imately 100-fold (235 vs. 2.5 μmol/l) in the absence of hepatocellular injury, as measured by biochemical and histological parameters [13]. This clinical profile differs from that observed in patients with progressive familial intrahepatic cholestasis (PFIC-2) where a defect in bile acid secretion, due to the loss of a bile salt efflux pump (Bsep) [14], results in elevated intracellular bile acid levels and considerable hepatocellular injury. The clinical profile presented by S-1 suggests the presence of a defect in bile acid uptake across the sinusoidal plasma membrane leading to elevated serum levels. Since NTCP mRNA and protein expression levels in S-1 were quantitatively normal with no defects in the deduced amino acid sequence [13], a goal of the present study was to characterize the expression of mEH. This analysis indicated that both mEH protein and mEH mRNA levels were substantially reduced, suggesting a specific defect in the promoter of the mEH gene (EPHX1) regulating mEH expression in human liver. Sequence analysis of EPHX1 from S-1 detected a point mutation in an upstream HNF-3 site (allele I) and in intron 1 (allele II) which significantly decreased EPHX1 promoter activity and altered transcription factor binding at these sites. These results demonstrate that these two allelic sites are involved in the regulation of EPHX1 expression and suggest that mEH may play a significant role in sodium-dependent bile acid uptake in human liver where its decreased expression may contribute to the etiology of hypercholanemia.

2. Materials and methods

2.1. Western blot analysis

Microsome preparations of liver biopsy specimens from S-1 and a normal subject were made as described [13]. Samples (2 μg) were separated on a 10% SDS-PAGE and subjected to Western analysis using a polyclonal antibody against mEH and the ProtoBlot alkaline phosphatase detection system (Promega, Madison, WI).

2.2. Northern blot analysis

Total RNA from biopsy samples from S-1 and a normal individual were isolated and blotted as previously described [13]. A 351 bp *Eco*RI (cDNA nt 808)/*Eco*RI (1159) fragment was isolated from a human mEH cDNA prepared by RT-PCR. This fragment, containing 67 bp of exon 5, 208 bp of exon 6, and 76 bp of exon 7, was used to detect the mEH message. This probe was radiolabeled using the random hexamer method. Hybridization and high stringency washes were performed according to manufacturer's instructions (New England Nuclear, Boston, MA). The blot was scanned using a phosphoimager (Molecular Dynamics, Sunnyvale, CA) and exposed to X-ray film with an intensifying screen at − 70 °C.

2.3. Cloning and sequencing of EPHX1

Genomic DNA was isolated from lymphocytes from S-1 and the patient's father using a Puregene DNA isolation kit (Gentra, Minneapolis, MN) using instructions supplied by the manufacturer. The sequences from − 2017 (the first nucleotide of exon 1 defined as + 1) to 3619, including 2017 bp 5′ flanking sequence, exon 1 (270 bp), intron 1 (3182 bp), the 5′ 167 bp of exon 2 were determined by sequencing [15] the overlapping DNA fragments produced by PCR using primers based on the EPHX1 gene sequence [16]. The genomic sequences more than 2 kb upstream from exon 1 were not included in the published EPHX1 gene sequence [16]. In order to clone and analyze these sequences, we obtained a P1 clone from Genomic Systems Inc. (St. Louis, MO), that contained the EPHX1 gene. The P1 clone was digested with *Hin*dIII and shotgun-ligated into PUC18. The *Hin*dIII clone containing the sequences upstream of − 2 kb was selected by restriction enzyme mapping for the 182 bp *Sca*I (− 1979)/*Hin*dIII (− 1797) fragment followed by confirmation by DNA sequencing. Based on the P1 sequence, the corresponding sequences up to − 4391 in S-1 and the father were determined.

2.4. Preparation of reporter gene constructs

To test the effect of the polymorphic forms in the 2 kb 5′ sequences, three different 1.3 kb *Stu*I(− 1298)/*Mfe*I(21) promoter fragments representing alleles I, II and III (wild-type) were cloned into the promoterless expression vector pOGH (Nichols Institute Diagnostics, San Juan Capistrano, CA) which contained a human growth hormone (hGH) reporter gene. To prepare the 1.3 kb constructs, a 1.3 kb *Stu*I/*Mfe*I fragment was isolated from the − 1528/310 fragment and reinserted into pBSK digested with *Eco*RV and *Eco*R1. The insert was then excised as a *Hin*dIII/*Bam*H1 fragment and cloned into the pOGH construct. To examine the effect of sequence variations upstream from the 2 kb 5′ -flanking region, a *Sst*I(− 4386)/*Mfe*I(21) fragment for each allele was used. To make these constructs, *Bam*HI (in primer)/*Hin*dIII(− 1797) fragments were isolated from the − 4391/− 967 PCR fragment and cloned into pBSK. The inserts were then isolated as *Xba*I(linker)/*Hin*dIII fragments and ligated to the *Hin*dIII(− 1797)/*Mfe*I(21) fragments of the corresponding alleles and the vector pOGH in which a *Xba*I site was added to the 5′ end of the original linker *Hin*dIII site. To examine the effect of the heterozygous sequence variations in intron 1 on the promoter activity, three different 5255 bp DNA fragments representing alleles I, II and III were inserted into the pOGH vector, including the 1797 bp 5′ sequence (from the *Hin*dIII site at − 1797), exon 1 (270 bp), entire intron 1 (3182 bp) and the first 6 bp of exon 2, including the nucleotide A of the translation initiation codon ATG (3458–3460). The 5255 bp DNA inserts for each allele were formed by ligation of four

subfragments for each allele: *Hin*dIII (−1797)/*Bgl*II(−1397) isolated from the −2017/−1387 fragment, *Bgl*II(−1397)/*Mfe*I(21) isolated from the −1528/310 fragment, *Mfe*I(21)/*Xba*I(2065) isolated from the −111/2331 fragment, and *Xba*I(2065)/3458 produced from the 1959/3619 fragment by PCR using the primer pair 5′-ATCAAGCTTCTTGG-GTTTCCAAGACAGAGCGAG-3′(5′ primer) and 5′-ATC*GGATCC*TGGCTCCTGCAAGAT-GAGGGGAAG-3′(3′ primer containing a *Bam*HI site). The resulting 5255 bp *Hin*dIII/*Bam*HI fragments were inserted into the pOGH vector.

2.5. Promoter activity assay

HepG2 and COS-7 cells were obtained from the American Type Culture Collection (ATCC) and grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum. The pOGH constructs were transiently transfected into HepG2 cells using the DEAE-Dextran transfection method [17]. Three micrograms of pSV-beta-galactosidase control vector (Promega) were included in each transfection as an internal control of transfection efficiency. The hGH produced was excreted into the medium and measured by a radioimmunoassay (Nichols Institute Diagnostics). Construct pXGH5, which contained the mouse metallothionein promoter, was used as a positive control to monitor transfection efficiency in each cell line. A promoterless construct was used as a negative control. The experiments ($n=3-4$) were carried out in triplicate on 100 mm dishes and the results were normalized to β-galactosidase values after subtracting the background of the negative control. The data represent the means ± S.E. Each separate experiment was repeated with freshly prepared plasmid preparations to eliminate the effect of DNA purity of each construct. Identical procedures were carried out using COS-7 cells.

2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from cultured HepG2 cells as previously reported [18]. HeLa nuclear extracts were purchased from Promega. Sense and antisense oligonucleotides containing the mutation sites were synthesized, annealed and radiolabeled with ^{32}P-dNTP in fill-in reactions with Klenow large fragment of DNA polymerase I. Two pairs of oligonucleotides were used to study the mutation site in intron 1 at nt 2557 on allele II. Only the sequence of sense oligonucleotides after fill-in reaction are shown below, with the nucleotides added during the fill-in reaction in parenthesis and the mutated nucleotides underlined. Sense strand for wild-type (WT); 5′-CATGGCTCT-TACACAGCTAAGCTGTGTGGC(CATG)-3′; C>G2557 (M); 5′-CATGGCTCTTACACAGCTAAG*G*TGTGTGGC(CATG)-3′. Three pairs of oligonucleotides were used to study the mutation site at nt −4238. Two pairs contained the HNF-3 site on allele I. Sense strand for wild-type; 5′-ATCTCTTATTTACTTTAAAAAA(CACT)-3′; mutation at −4238T>A; 5′-ATCTCTTATTTACTT*A*AAAAAA(CACT)-3′. Oligonucleotides containing a consensus HNF-3 binding site [19] were also synthesized. The sense strand was 5′-AAAGAGATTATTTGCTTATTG (CATCGA)-3′; 1.25 μg of poly[dI–dC].poly[dI–dC] (Sigma, St. Louis, MO) was preincubated with 5 μg of nuclear proteins for 10 min at room temperature in a mix of 19 μl containing 20 mM Tris–HCl, pH 7.8, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT and 10% glycerol. Radiolabeled oligonucleotides (0.5 μl) (about 10 fmol and 10,000 cpm) were added and the incubation was continued for another 20 min at room temperature. After mixing with 2.5 μl of loading buffer (250 mM Tris–HCl, pH 7.8, 0.2% bromophenyl blue, 40% glycerol), the mixture was loaded on a 6% nondenaturing acrylamide (acrylamide:bisacrylamide = 29:1) in 0.5 × Tris–borate/EDTA electrophoresis buffer prerun at 50 V for 1 h. Gel electrophoresis was carried out at 150 V (about 10 V/cm) for 1.5 h. The bands were visualized by autoradiography. To further characterize the factor binding to the allele I site, antibodies against HNF-3α, β and γ were incubated with the HepG2 nuclear extract for 2 h on ice before adding the radiolabeled probe. EMSA was then performed as described above.

2.7. Genotyping with allele-specific PCR

In order to estimate the allele frequency of the −4238T>A mutation at the upstream HNF-3 site and the 2557C>G mutation in intron 1, semi-nested allele-specific PCR was performed as previously described [20] but with primers specific to both alleles which differ only at the last (3′) nucleotide.

2.8. Human subjects

Informed consent in writing was obtained from each subject and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review committee.

3. Results

3.1. Western blot analysis of mEH expression

Western blot analyses of mEH expression were performed with microsome preparations from a liver biopsy sample obtained from S-1 and two normal subjects. As shown in Fig. 1, the level of mEH expression in the patient (S-1) was reduced to less than 5% of the levels observed in normal liver samples, suggesting that the greatly decreased levels of mEH may contribute to the observed elevated serum bile salt levels.

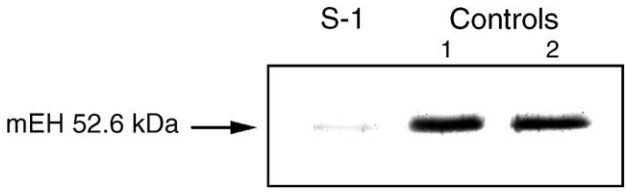

Fig. 1. Analysis of mEH expression in the liver of S-1 and two control individuals. Two micrograms of the microsome preparation from liver biopsy samples were analyzed by SDS-PAGE and immunoblotted with a polyclonal antibody against mEH and the ProtoBlot alkaline phosphatase detection system (Promega).

3.2. Northern blot analysis of mEH mRNA

Northern blot analyses on total mRNA were performed to establish whether the low level of mEH expression observed in the patient's (S-1) liver was associated with reduced mEH mRNA levels. A DNA probe containing exon 6 and part of exons 5 and 7 were isolated for Northern blot analysis. As shown in Fig. 2, the mEH mRNA level was decreased to approximately 15% of normal levels suggesting that the low mEH levels observed in S-1 may result from a defect in the transcription of EPHX1.

3.3. Sequencing of the 5′ flanking region and intron 1 of EPHX1

Sequence analysis was carried out in an effort to characterize possible polymorphisms that could inhibit EPHX1 transcriptional activity resulting in the low levels of mEH mRNA. The strategies for cloning the genomic sequences are shown in Fig. 3. In the first 2 kb of the 5′ region, five heterozygous sequence variations were observed in allele I from S-1 and the patient's father, and allele II from S-1

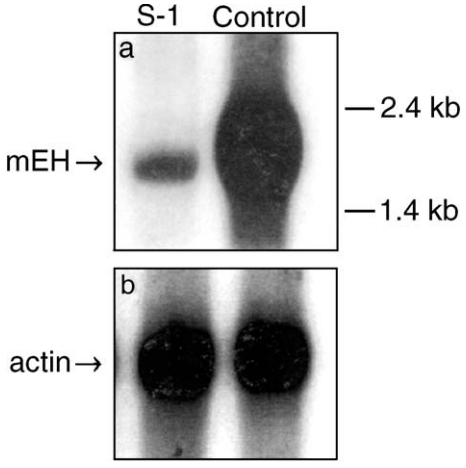

Fig. 2. Northern analysis of mEH mRNA levels in the liver of S-1 and a control individual. Ten micrograms of total RNA isolated from liver biopsy samples were hybridized with a cDNA probe containing exons 5, 6, and 7 (a). Panel (b) shows the same blot probed by an actin cDNA probe to normalize the intensities of the mEH signals.

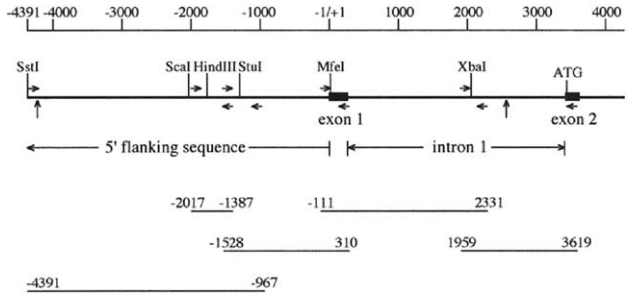

Fig. 3. The strategy for cloning and sequencing EPHX1 genomic sequences. The scale on the top shows base pair numbers of the EPHX1 gene. The sequences upstream of the transcription initiation site are shown in negative numbers. The EPHX1 gene is represented by a thick line and exons 1, 2, represented by closed boxes. The horizontal arrows represent PCR primers. The thin lines below are PCR products. Vertical arrows indicate the location of allele I (− 4238T>A) and II (2557C>G) mutations shown in Table 1.

when compared with the wild-type sequence (N) as shown in Table 1: (a) an A insertion at − 1060, and a − 718C>A substitution in allele I of S-1 and the father; (b) three substitutions (− 200T>C, − 259T>C and − 290G>T) in allele II in S-1. The sequence upstream from − 2 kb was not available and was thus obtained using a P1 clone with an approximately 100-kb insert containing EPHX1. A *Hin*dIII/*Hin*dIII P1 fragment containing the region immediately upstream from the 5′ end of the published sequence [16] was selected by restriction enzyme mapping, sequenced and designated as N (Table 1). The corresponding sequences from the patient and the father were cloned by PCR and compared with the P1 sequence. Allele I of the patient's DNA in this region was determined by the A insertion at − 1060, which was included in the PCR product (− 4391/

Table 1
Sequence variation in EPHX1 in the 5′ upstream and intron regions in the subject (S-1) and father (F/S-1)

Position	N	S-1		F/S-1	
		allele type		allele type	
		I	II	I	III
-4238 (146724)	T	**A**	T	**A**	T
-2324 (148639)	A	-	A	-	A
-2323 (148640)	A	-	A	-	A
-2322 (148641)	C	-	C	-	C
-1060 (149903)	-	**A**	-	**A**	-
-718 (150246)	C	**A**	C	**A**	C
-290 (150615)	G	G	**T**	G	G
-259 (150705)	T	T	**C**	T	T
-200 (150764)	T	T	**C**	T	T
+880 (151845)	T	**C**	T	**C**	T
+2140 (153104)	G	**A**	G	**A**	G
+2557 (153520)	C	C	**G**	C	C

N denotes wild-type sequences obtained from a P1 clone (Materials and methods) (lines 1 to 4) and [16] (lines 5 to 12). The sequences differing from N are in boldface. The numbers in parentheses are the corresponding positions in GenBank sequences AC058768. A dash (–) in N denotes an insertion at that position and a dash in S-1 and the F/S-1 denotes a deletion. The two sequence variations which affect promoter activity are boxed.

−967, Fig. 3). The absence of this insertion identified the patient's DNA as allele II. No sequence variations were detected in allele II compared with the control sequence (N), however, a −4238T>A at a putative HNF-3 site [19] and a AAC deletion at −2322, −2323 and −2324 were detected in allele I (Table 1). Sequencing of the 3179 bp intron 1 of EPHX1 indicated the presence of three heterozygous substitutions in S-1 and the father, namely, a 880 T>C and 2140 G>A on allele I of both subjects and a 2557 C>G substitution on allele II of S-1.

3.4. Promoter activity measurements

To test the effects of the sequence polymorphisms observed in the 4 kb 5′ flanking region and in intron 1 of EPHX1 on promoter activity, fragments from these regions, representing alleles I, II and III, were linked to the hGH reporter gene in the expression vector pOGH and transiently transfected into HepG2 cells in which endogenous mEH expression could be detected by Western analysis. The five heterozygous sequence polymorphisms in the −200 to −1060 bp region (Table 1) had no effect on EPHX1 promoter activity (Fig. 4C). In contrast, reporter constructs using allele I, which included the AAC deletion (−2322, −2323, −2324) and the −4238T>A substitution demonstrated a significant decrease in promoter activity (Fig. 4B). This effect could be obtained utilizing a construct that contained only the −4238T>A mutation.

Analysis of intron 1 polymorphisms demonstrated that two of the heterozygous substitutions on allele I 880 T>C and 2140G>A had no effect on promoter activity (Fig. 5B); however, the 2557 C>G substitution in allele II significantly suppressed activity by approximately 86%. A similar result was also obtained in the absence of the other 5′ polymorphisms (Fig. 5B). Transfection of the wild-type intron 1 and the 5′ upstream constructs into COS-7 cells did not result in measurable promoter activity (data not shown), suggesting that the observed EPHX1 promoter activity in HepG2 cells may be liver-specific. These results therefore demonstrate that a mutation in each allele of EPHX1 in S-1 leads to a significant decrease in promoter activity, suggesting that these mutations may affect mEH expression in the hepatocytes of S-1.

3.5. Electrophoretic mobility shift assay

EMSA analysis was employed to evaluate the effect of the two mutations (2557C>G, −4238T>A) in alleles II and I, which significantly inhibit EPHX1 promoter activity, on the nuclear protein binding patterns at putative transcription factor binding sites. EMSA analysis using a radiolabeled wild-type oligonucleotide (2540–2569 bp)

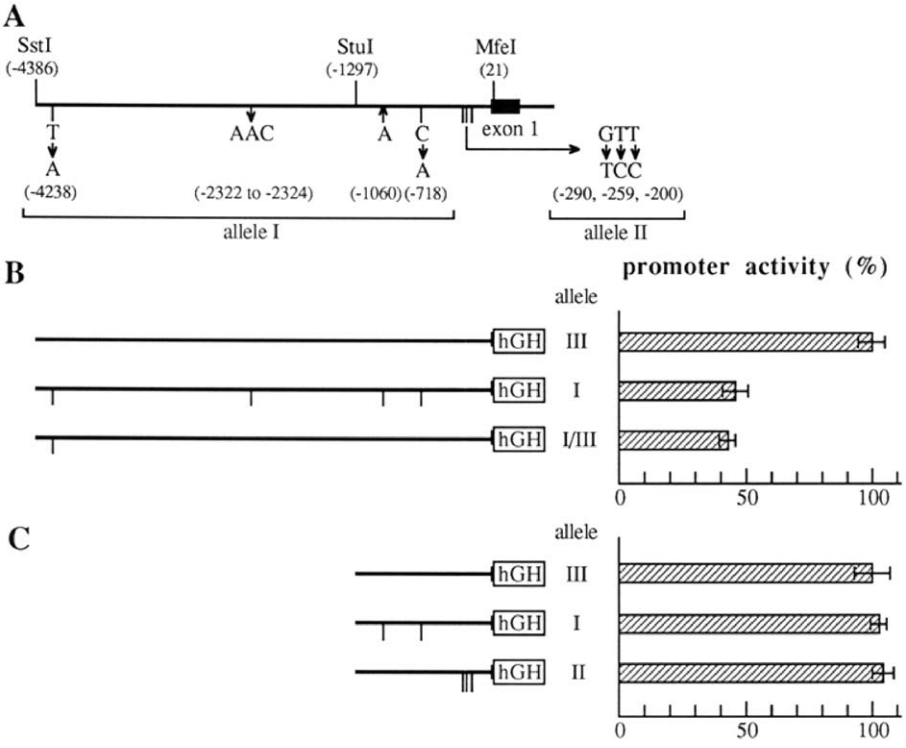

Fig. 4. The effect of the sequence variations in the 5′ flanking region of S-1 on EPHX1 promoter activity. Six pOGH constructs representing alleles I, II and III were transiently transfected into HepG2 cells and the released hGH measured by a radioimmunoassay. (A) Sequence variations in each allele and the DNA fragments used in the promoter activity assay in B (4.4 kb *Sst*I/*Mfe*I) and C (1.3 kb *Stu*I/*Mfe*I). (B) DNA fragments of each allele connected to the hGH reporter gene with corresponding sequence variations. The activity measured is expressed as the percentage of the wild-type (allele III). A I/III construct was used to evaluate only the effect of the −4238T>A mutation in allele I. (C) The three constructs were analyzed for promoter activity as described in B.

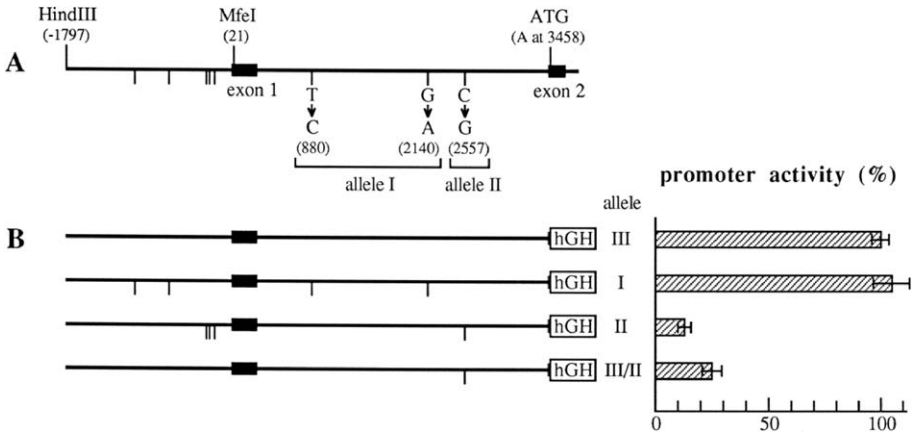

Fig. 5. The effect of sequence variations in intron 1 of S-1 on EPHX1 promoter activity. Four pOGH constructs were analyzed as describe in Fig. 4. (A) DNA region (*Hin*dIII/A of ATG in exon 2) to be used in the promoter activity assay, with the sequence variations in allele I and II indicated. (B) DNA fragments of each allele were connected to the hGH reporter gene. A III/II constructs was also made in which the 5′ flanking sequence of the allele II was replaced by that of the allele III (wild-type) so the effect of the 2557C>G mutation in allele II could be examined alone. Analysis of promoter activity was carried out as described in Fig. 4.

and a nuclear extract from HepG2 cells resulted in the formation of one band (Fig. 6, lane 1). This binding could be inhibited in a concentration-dependent fashion with an excess (10−100×) of unlabeled probe demonstrating the specificity of the interaction (lanes 2−4). Competition with the unlabeled probe containing the 2557C>G mutation, however, had no effect on the binding (lane 5). EMSA analysis with the mutant oligonucleotide resulted in the total loss of the labeled band observed with the WT probe (lane 6). The small amount of binding was shown to be non-specific as it was unaffected by unlabeled probe (lane 7).

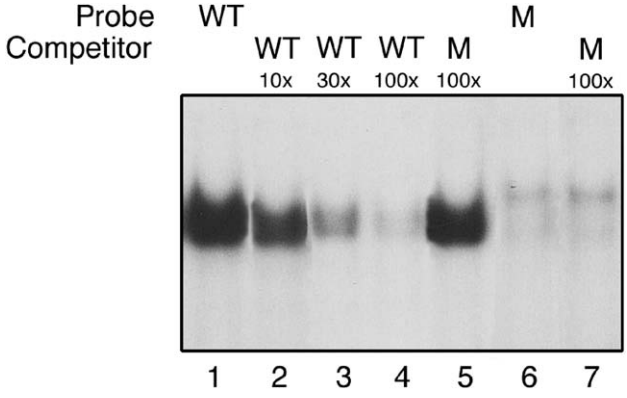

Fig. 6. Characterization of nuclear protein binding to the intron 1 region containing the allele II 2557C>G mutation site by EMSA. Radiolabeled oligonucleotides (10,000 cpm) containing the wild-type (WT) and mutant (M) sequences (Materials and methods) were used as probes in EMSA using HepG2 nuclear extracts (5 μg). A 10- to 100-fold excess of unlabeled oligonucleotide was used as competitive DNA. Nuclear proteins bound to the WT oligonucleotide in the absence (lane 1) or presence (lane 2−4) of unlabeled WT oligonucleotide or M oligonucleotide (lane 5). Nuclear proteins labeled with the oligonucleotide containing the naturally occurring 2557C>G mutation (M) in the absence (lane 6) or presence (lane 7) of 100-fold excess of unlabeled oligonucleotide (M).

Examination of the region surrounding the −4238T>A upstream mutation on allele I indicated the presence of a consensus HNF-3 binding site [19]. EMSA analysis with the radiolabeled wild-type probe produced two protein−DNA species (A,B) (Fig. 7, lane 1). The specificity of this interaction was demonstrated by competition with unlabeled probe (lane 2). Competition with an HNF-3 oligonucleotide also inhibited the formation of bands A and B (lane 3). EMSA analysis using the probe containing the −4238T>A mutation (M) resulted in a substantial specific increase in the intensity of bands A and B (lane 5). This binding was shown, as in the wild-type case, to be specific since it could be eliminated by competition with unlabeled probe (lane 6) or with an HNF-3 oligonucleotide (lane 7). To further establish the presence of HNF-3 in the protein−DNA complex, anti HNF-3 antibodies were incorporated into

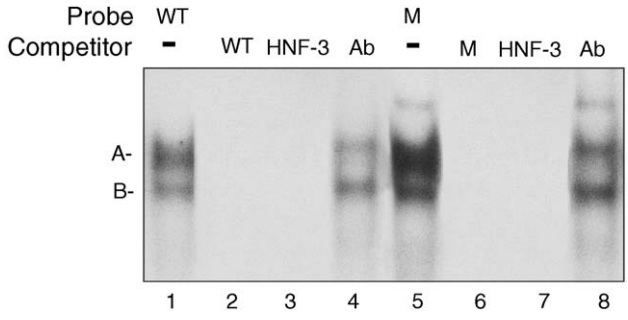

Fig. 7. Characterization of nuclear protein binding to the 5′ upstream region containing the allele I −4238T>A mutation site by EMSA. Radiolabeled oligonucleotides (10,000 cpm) containing the WT and M sequences (Materials and methods) were used as probes in EMSA as described in Fig. 6. Nuclear proteins bound to the WT oligonucleotide alone (lane 1) and in the presence of 50-fold excess of unlabeled WT oligonucleotide (lane 2), HNF-3 (lane 3) or HNF-3β antibody (Ab) (lane 4). Nuclear proteins bound to the oligonucleotide containing the −4238T>A mutation (M) (lane 5) and in the presence of a 50-fold excess of unlabeled oligonucleotide (M) (lane 6), HNF-3 (lane 7) or HNF-3β antibody (lane 8).

the EMSA analysis. When an anti-HNF-3β antibody was added to the nuclear extract reaction, a substantial reduction in the intensity of band A was observed for both the wild-type and mutant forms (lanes 4, 8). No change in the binding was observed when anti-HNF-3α or γ antibodies were used (data not shown). The decrease in band A intensity using the WT and M probes suggests specific binding of HNF-3β to the added antibody. The absence of an observable supershifted band suggests that the resulting complex now binds very weakly to the labeled oligonucleotide as previously reported [21].The complexes shown in Figs. 6 and 7 were not observed when HeLa nuclear extracts were used (data not shown). The EMSA analysis thus supports the thesis that the mutations in the HNF-3 region of allele I and in intron 1 of allele II can affect EPHX1 promoter activity by altering the binding characteristics of transcription factors that bind in these two regions.

3.6. Genotyping with allele-specific PCR

Allele-specific PCR was performed to determine the frequency of occurrence of the EPHX1 mutation described above in the general population composed of Caucasians, African–Americans, Latinos and Asians [22]. Serum bile acid concentration values are not available. The −4238T>A mutation in allele I was found as a heterozygous change in 0.7% of the subjects ($n=270$). The 2557C>G mutation in allele II was found as a heterozygous change in 16% and as a homozygous change in 1.6% of the subjects ($n=509$).

4. Discussion

In this study, we have established that the expression of the bile acid transporter, mEH, is greatly reduced in a human subject (S-1) who exhibits highly elevated serum bile acid levels (hypercholanemia) in the absence of cholestatic parameters, possibly resulting from an apparent reduced bile acid uptake capacity. Northern blot analysis demonstrated that mEH mRNA was greatly decreased, suggesting the presence of a mutation(s) that affected EPHX1 promoter activity. Sequence analysis of the 4.4 kb 5′ upstream and 3.2 kb intron 1 regions elucidated a number of polymorphisms (Table 1) whose effect on EPHX1 promoter activity were evaluated using a transient expression assay in HepG2 cells. A −4238T>A substitution in the 5′ upstream region in allele I at a putative HNF-3 site and a 2557C>G substitution in intron 1 of allele II both significantly decreased EHPX1 promoter activity (Figs. 4 and 5). The 86% decrease in promoter activity resulting from the intron 1 mutation is consistent with the approximately 85% decrease in mEH mRNA. The 53% decrease resulting from the −4238T>A mutation, however, may be an underestimate of the actual effect in human hepatocytes since additional regulatory *cis*-elements may be missing from the constructs used in this analysis and transcription factor levels found in HepG2 cells may be different from those found in hepatocytes. Many promoters have tissue-specific regulatory elements located far upstream from the transcription initiation site and/or in introns, where mutations or deletions result in significant alterations in promoter activity [23–25] as described herein for EPHX1. The existence of both defective alleles appears necessary for the expression of the observed hypercholanemia since the father of S-1, who only possessed the functional mutation in the HNF-3 region, had normal serum glycocholate levels.

EMSA were performed with HepG2 nuclear extracts to determine transcription factor-binding elements in the mutated regions of allele I and allele II. Specific binding of nuclear proteins was detected at both sites, which was significantly altered as a result of the observed mutations, suggesting that these sites are important regulatory regions in EPHX1 whose alteration results in significant decreases in EPHX1 promoter activity. The presence of the −4238T>A mutation resulted in a large increase in HNF-3β binding (Fig. 7). These results suggest that binding of this factor could act as a repressor of EPHX1 expression, an effect further increased as a result of the mutation. HNF-3β has also been shown to negatively regulate glucagon gene expression [26]. HFN-3 is a member of the winged helix family of transcription factors and plays a critical role in liver-specific expression of many genes by binding to the promoter region [27]. The presence of the 2557C>G mutation in intron 1, in contrast, resulted in the loss of binding of, as yet, an uncharacterized factor (Fig. 6, lane 6) suggesting that this protein plays a significant role in EPHX1 transcription. Although the sequence in this region is similar to an AP-4 site, the intron 1 probe was unable to bind any factors in HeLa nuclear extract although it contained ample AP-4 as evidenced by the binding to the authentic AP-4 oligonucleotides (data not shown). The identity of the factor(s) binding to this region is under investigation.

The concentration of bile acids in the blood is regulated by the coordinated activity of the transport systems for these substrates in hepatocytes and ileal enterocytes [28]. Several cholestatic disorders have been associated with alterations in hepatocellular transport systems in the canalicular membrane such as progressive familial intrahepatic cholestasis Type 1 [29], Type 2 [14], Type 3 [30], and benign recurrent intrahepatic cholestasis [29] where liver marker enzymes are highly elevated accompanied by significant morphological alterations indicating hepatocellular injury. The report [13] of a patient with highly elevated levels of bile acids in the blood with no significant hepatocellular injury suggests that this disease, in contrast to the above cholestatic disorders, may result from a defect in bile acid uptake across the sinusoidal membrane. While the expression levels and amino acid sequence of the sodium-dependent bile acid uptake protein NTCP were normal in this subject (S-1), the level of mEH expressed in the liver was reduced by approximately 95%, suggesting a significant role for mEH

in the etiology of the syndrome observed in this patient and in bile acid transport in human liver. The normal levels of NTCP in S-1 are in contrast to the observation in a rat model of cholestasis secondary to common bile duct ligation [11] and in biliary atresia patients [13] where extensive down-regulation of NTCP and NTCP mRNA were observed in cases where intrahepatocyte bile acid levels are significantly elevated. The failure to observe NTCP down-regulation in S-1 suggests that intracellular bile acid levels are not elevated, and supports the proposal that the observed hypercholanemia results from an uptake rather than an efflux defect.

This conclusion is further supported utilizing the mEH knockout mouse [31]. In our studies, analysis of the serum by electrospray tandem mass spectrometry indicated that the concentration of several serum bile acids were substantially increased. This observation is consistent with the bile acid transport properties of mEH previously reported [3–9]. In addition, the expression of several other genes involved in the synthesis and transport of bile acids were also shown to be significantly altered. The results of these studies, which will be described in a subsequent report, strongly suggest a role of mEH in bile acid homeostasis. In this regard, it should also be noted that feeding mice a diet supplemented with 1% cholic acid resulted in a 4-fold increase in the expression of mEH mRNA [32].

The contributions of mEH and NTCP to bile acid transport in humans depends on the substrate specificities of these two transport proteins. While NTCP is able to transport taurocholate, its ability to transport glycocholate is significantly reduced, with levels ranging from 30% of taurocholate values in transfected CHO cells [33] to 14% in transfected COS-7 cells (unpublished observation). In contrast, mEH expressed in MDCK cells was shown to transport glycocholate at a rate 2.5 times greater than that observed for taurocholate uptake (unpublished observation). Because glyco-conjugation is the major form of bile acid in humans [34–36], the above results suggest that mEH may be responsible for a significant percentage of bile acid uptake in human liver such that the loss of mEH in this patient (S-1), despite normal NTCP expression, appears to result in a significant decrease in net bile acid uptake resulting in elevated serum bile acid levels. The severity of this hypercholanemic syndrome should therefore also depend on the bile acid conjugation profile of a subject expressing these mutations where a higher percentage of tauro-conjugation could attenuate the elevated serum bile acid levels as they would be efficiently transported by NTCP. The co-expression of two functionally distinct hepatocyte sodium-dependent bile acid transport systems in humans is also supported by the differential inhibition of taurocholate and glycocholate by cyclosporin A [37].

The central role of mEH in the metabolism of numerous carcinogens suggests that variations in mEH levels and/or effective activity could have a profound effect on the susceptibility to various forms of cancer. This hypothesis is supported by our recent study that demonstrates an association between several polymorphic forms of EPHX1 including the intron 1 mutation described in this report and susceptibility to colorectal adenomas [22], an observation further supporting the effect of the 2557C>G mutation on the expression levels of mEH. The role of mEH in the metabolic activation of polycyclic aromatic hydrocarbon carcinogens has also been established utilizing the mEH knockout mouse [31].

In conclusion, these studies have demonstrated that two point mutations in regulatory regions of EPHX1, found in a patient with highly elevated serum bile acids (hypercholanemia) result in an extensive reduction in gene expression associated with a large increase in factor binding at an HNF-3 site in allele I and the loss of factor binding at the intron 1 site in allele II. These DNA alterations are associated with an apparent decrease in bile acid transport capacity, which in turn decreases intracellular bile acids which play a critical role in cholesterol homeostasis in addition to numerous physiological processes. Further analysis of the factors that bind to the intron 1 region, the 5′-upstream region as well as the basal promoter is in progress, in order to elucidate the mechanism of EPHX1 regulation. The transport characteristic of mEH and NTCP in relation to the bile acid conjugation profiles in humans as well as the results of this study suggest that mEH may play a significant role in human hepatocyte bile acid uptake and in the cause of the observed hypercholanemia.

Acknowledgements

This work was supported by Grants DK 25836 (D.L.), USC Research Center for Liver Disease Grant DK 48522 (Q.-S.Z) and DK 54165 (B.L.S.) from the National Institutes of Health. We wish to thank Dr. Andrew Stolz, Dr. Neil Kaplowitz, Dr. Amy Lee, Dr. Robert Stellwagen, Dr. Michael Stallcup and Dr. Gerhard Coetzee for useful discussions and critical review of this manuscript, Dr. S. Duncan (Rockefeller University) for the anti-HNF-3γ antibody and Dr. R. Costa (University of Illinois) for anti-HNF-3α and β antibodies, and Ahn Diep for genotyping analysis.

References

[1] A.J. Fretland, C.J. Omiecinski, Epoxide hydrolases: biochemistry and molecular biology, Chem. Biol. Interact. 129 (1997) 41–59.
[2] Q. Zhu, P. von Dippe, W. Xing, D. Levy, Membrane topology and cell surface targeting of microsomal epoxide hydrolase. Evidence for multiple topological orientations, J. Biol. Chem. 274 (1999) 27898–27904.
[3] M. Ananthanarayanan, P. von Dippe, D. Levy, Identification of the hepatocyte Na^+-dependent bile acid transport protein using monoclonal antibodies, J. Biol. Chem. 263 (1988) 8338–8343.
[4] P. von Dippe, D. Levy, Characterization of the bile acid transport system in normal and transformed hepatocytes. Photoaffinity label-

ing of the taurocholate carrier protein, J. Biol. Chem. 258 (1983) 8896–8901.
[5] P. von Dippe, P. Drain, D. Levy, Synthesis and transport chracteristics of photoaffinity probes for the hepatocyte bile acid transport system, J. Biol. Chem. 258 (1983) 8890–8895.
[6] P. von Dippe, D. Levy, Reconstitution of the immunopurified 49-kDa sodium-dependent bile acid transport protein derived from hepatocyte sinusoidal plasma membranes, J. Biol. Chem. 265 (1990) 14812–14816.
[7] P. von Dippe, D. Levy, Expression of the bile acid transport protein during liver development and in hepatoma cells, J. Biol. Chem. 265 (1990) 5942–5945.
[8] P. von Dippe, M. Amoui, C. Alves, D. Levy, Na^+-dependent bile acid transport by hepatocytes is mediated by a protein similar to microsomal epoxide hydrolase, Am. J. Physiol. 264 (1993) G528–G534.
[9] P. von Dippe, M. Amoui, R.H. Stellwagen, D. Levy, The functional expression of sodium-dependent bile acid transport in Madin–Darby canine kidney cells transfected with the cDNA for microsomal epoxide hydrolase, J. Biol. Chem. 271 (1996) 18176–18180.
[10] B. Hagenbuch, B. Stieger, M. Foguet, H. Lubbert, P.J. Meier, Functional expression cloning and characterization of the hepatocyte Na^+/bile acid cotransport system, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 10629–10633.
[11] C. Gartung, M. Ananthanarayanan, M.A. Rahman, S. Schuele, S. Nundy, C.J. Soroka, A. Stolz, F.J. Suchy, J.L. Boyer, Down-regulation of expression and function of the rat liver Na^+/bile acid cotransporter in extrahepatic cholestasis, Gastroenterology 110 (1996) 199–209.
[12] L. Meerman, N.R. Koopen, V. Bloks, H. van Goor, R. Havinga, B.G. Wolthers, W. Kramer, S. Stengelin, M. Muller, F. Kuipers, P.L. Jansen, Biliary fibrosis associated with altered bile composition in a mouse model of erythropoietic protoporphyria, Gastroenterology 117 (1999) 696–705.
[13] B.L. Shneider, V.L. Fox, K.B. Schwarz, C.L. Watson, C.M. Ananthanarayanan, S. Thevananther, D.M. Christie, W. Hardikar, K.D.R. Setchell, G. Mieli-Vergani, F.J. Suchy, A.P. Mowat, Hepatic basolateral sodium-dependent-bile acid transporter expression in two unusual cases of hypercholanemia and in extrahepatic biliary atresia, Hepatology 25 (1997) 1176–1183.
[14] S.S. Strautnieks, L.N. Bull, A.S. Knisely, S.A. Kocoshis, N. Dahl, H. Arnell, E. Sokal, K. Dahan, S. Childs, V. Ling, M.S. Tanner, A.F. Kagalwalla, A. Nemeth, J. Pawlowska, A. Baker, G. Mieli-Vergani, N.B. Freimer, R.M. Gardiner, R.J. Thompson, A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis, Nat. Genet. 20 (1998) 233–238.
[15] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 411–416.
[16] C. Hassett, K.B. Robinson, N.B. Beck, C.J. Omiecinski, The human microsomal epoxide hydrolase gene (EPHX1): complete nucleotide sequence and structural characterization, Genomics 23 (1994) 433–442.
[17] V. Esser, L.E. Limbird, M.S. Brown, J.L. Goldstein, D.W. Russell, Mutational analysis of the ligand binding domain of the low density lipoprotein receptor, J. Biol. Chem. 263 (1988) 13282.
[18] J.D. Dignam, R.M. Lebovitz, R.G. Roeder, Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei, Nucleic Acids Res. 11 (1983) 1475–1489.
[19] C.D. Bingle, J.D. Gitlin, Identification of hepatocyte nuclear factor-3 binding sites in the Clara cell secretory protein gene, Biochem. J. 295 (1993) 227–232.
[20] H. Schuster, G. Rauh, S. Muller, C. Keller, G. Wolfram, N. Zollner, Allele-specific and asymmetric polymerase chain reaction amplification in combination: a one step polymerase chain reaction protocol for rapid diagnosis of gamilial defective apolipoprotein B-100, Anal. Biochem. 204 (1992) 22–25.
[21] F. Beurton, U. Bandyopadhyay, B. Dieumegard, R. Barouki, M. Aggerbeck, Delineation of the insulin-responsive sequence in the rat cytosolic aspartate aminotransferase gene: binding sites for hepatocyte nuclear factor-3 and nuclear factor I, Biochem. J. 343 (1999) 687–695.
[22] V. Cortessis, K. Siegmund, Q. Chen, N. Zhou, A. Diep, H. Frankl, E. Lee, Q. Zhu, R. Haile, D. Levy, A case-control study of microsomal epoxide hydrolase, smoking, meat consumption, glutathione S-transferase M3, and risk of colorectal adenomas, Cancer Res. 61 (2000) 2381–2385.
[23] H. Thomassin, B. Bois-Joyeux, R. Delille, R. Ikonomova, J.L. Danan, Chicken ovalbumin upstream promoter–transcription factor, hepatocyte nuclear factor 3, and CCAAT/enhancer binding protein control the far-upstream enhancer of the rat alpha-fetoprotein gene, DNA Cell Biol. 5 (1996) 1063–1074.
[24] M. Nakajima, T. Yokoi, M. Mizutani, M. Kinoshita, M. Funayama, T. Kamataki, Genetic polymorphism in the 5′-flanking region of human CYP1A2 gene: effect on the CYP1A2 inducibility in humans, J. Biochem. 125 (1999) 803–808.
[25] C.J. Storbeck, L.A. Sabourin, J.D. Waring, R.G. Korneluk, Definition of regulatory sequence elements in the promoter region and the first intron of the myotonic dystrophy protein kinase gene, J. Biol. Chem. 273 (1998) 9139–9147.
[26] J. Philippe, C. Morel, V.R. Prezioso, Glucagon gene expression is negatively regulated by hepatocyte nuclear transcription factor 3β, Mol. Cell. Biol. 14 (1994) 3514–3523.
[27] S. Cereghini, Liver-enriched transcription factors and hepatocyte differentiation, FASEB J. 10 (1996) 267–282.
[28] R.J. Bahar, A. Stolz, Bile acid transport, Gastroenterol. Clin. North Am. 28 (1999) 27–58.
[29] L.N. Bull, M.J. van Eijk, L. Pawlikowska, J.A. DeYoung, J.A. Juijn, M. Liao, L.W. Klomp, N. Lomri, R. Berger, B.F. Scharschmidt, A.S. Knisely, R.H. Houwen, N.B. Freimer, A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis, Nat. Genet. 18 (1998) 219–224.
[30] J.M. de Vree, E. Jacquemin, E. Sturm, D. Cresteil, P.J. Bosma, J. Aten, J.F. Deleuze, M. Desrochers, M. Burdelski, O. Bernard, R.P. Oude Elferink, M. Hadchouel, Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 282–287.
[31] M. Miyata, G. Kudo, Y.-H. Lee, T.J. Yang, H.V. Gelboin, P. Fernandez-Salguero, S. Kimura, F. Gonzalez, Targeted disruption of the microsomal epoxide hydrolase gene, J. Biol. Chem. 274 (1999) 23963–23968.
[32] C.J. Sinal, M. Tohkin, M. Miyaa, J.M. Ward, G. Lambrt, F. Gonzalez, Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis, Cell 102 (2000) 731–744.
[33] A. Schroeder, U. Eckhardt, B. Stieger, R. Tynes, C.D. Schteingart, A.F. Hofmann, P.J. Meier, B. Hagenbuch, Substrate specificity of the rat liver Na(+)-bile salt cotransporter in *Xenopus laevis* oocytes and in CHO cells, Am. J. Physiol. 274 (1998) G370–G375.
[34] G. Hedenborg, A. Norman, Fasting and postprandial serum bile acid concentration with special reference to variations in the conjugate profile, Scand. J. Clin. Lab. Invest. 45 (1985) 151–156.
[35] H.J. Wildgrube, H. Stockhausen, J. Petri, U. Fussel, H. Lauer, Naturally occurring conjugated bile acids, measured by high-performance liquid chromatography in human, dog, and rabbit bile, J. Chromatogr. 353 (1986) 207–213.
[36] S. Perwaiz, B. Tuchweber, D. Magnault, T. Gilat, I.M. Yousef, Determination of bile acids in biological fluids by liquid chromatography-electrospray tandem mass spectrometry, J. Lipid Res. 42 (2001) 114–119.
[37] S.A. Azer, N.H. Stacey, Differential effects of cyclosporin A on the transport of bile acids by human hepatocytes, Biochem. Pharmacol. 46 (1993) 813–819.